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The *H19* Differentially Methylated Region Marks the Parental Origin of a Heterologous Locus without Gametic DNA Methylation

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***Igf2* and *H19* are coordinately regulated imprinted genes physically linked on the distal end of mouse chromosome 7. Genetic analyses demonstrate that the differentially methylated region (*DMR*) upstream of the *H19* gene is necessary for three distinct functions: transcriptional insulation of the maternal *Igf2* allele, transcriptional silencing of paternal *H19* allele, and marking of the parental origin of the two chromosomes. To test the sufficiency of the *DMR* for the third function, we inserted *DMR* at two heterologous positions in the genome, downstream of *H19* and at the *alpha-fetoprotein* locus on chromosome 5. Our results demonstrate that the *DMR* alone is sufficient to act as a mark of parental origin. Moreover, this activity is not dependent on germ line differences in *DMR* methylation. Thus, the *DMR* can mark its parental origin by a mechanism independent of its own DNA methylation.**

The *H19* and *Igf2* genes are part of a cluster of imprinted genes on the distal end of mouse chromosome 7. The genome organization and regulation of the genes in this cluster are highly conserved on chromosome 11p15.5 in humans (32–34). IGF2 is a potent fetal mitogen (8, 31), and loss of imprinting mutations that result in increased *IGF2* expression are associated with Beckwith-Wiedemann syndrome and with several types of tumors (12, 13, 30, 47). The biological function of the *H19* gene product is less clear. Recent studies have suggested that Wilms' tumors frequently associated with Beckwith-Wiedemann syndrome are more likely when the loss of imprinting includes *H19* in addition to *IGF2* (7). These results are consistent with those of earlier cell culture studies suggesting that the *H19* RNA might function as a tumor suppressor (17).

The monoallelic expression of the *H19* and *Igf2* genes is dependent on a common *cis*-acting regulatory element, the *DMR* (for differentially methylated region) located between kb −4.4 and −2 upstream of the *H19* promoter (23, 44) (Fig. 1A). This element contains a transcriptional insulator that prevents activation of the *Igf2* promoters by the shared enhancers located downstream of the *H19* gene. When paternally inherited, the *DMR* sequence is methylated and insulator activity is blocked so that *Igf2* expression is permitted (2, 19, 23-25, 41). At the same time, the methylated paternal *DMR* induces epigenetic changes at the *H19* promoter that silence *H19* expression (4, 9, 40). These epigenetic changes are developmentally programmed, and once established, they can maintain repression of the paternal *H19* even in the absence of the *DMR* (39, 40).

Beyond the *DMR*'s role in regulating transcription of the two genes, genetic evidence is consistent with the notion that the *DMR* has a third distinct function: it is at least part of the imprinting control element (*ICE*) for the *H19* and *Igf2* genes. That is, the element appears to be necessary for marking the chromosomal origin of the two genes and of *H19* transgenes (11, 23, 44). Furthermore, molecular studies have shown that the *H19DMR* is methylated in sperm but not in oocytes and the differential methylation is maintained during the global changes in methylation patterns associated with early development (3, 14, 45). These findings suggest that differential methylation of the *DMR* is probably the primary mark for the imprinting of the *Igf2*/*H19* locus and, likewise, that the *DMR* sequences are those that contain the original or primary epigenetic mark distinguishing the maternal and paternal chromosomes. In this study, we determined that the *DMR* is sufficient to mark the parental origins of normally nonimprinted loci. However, this activity is not always dependent on germ line differences in methylation of *DMR* sequences.

MATERIALS AND METHODS

Generation of mutant mice. All animal research was conducted in full accord with the requirements of the NICHD Animal Care and Use Committee. To generate *H19R* and *H19F*, the *H19DMR* carried on a 2.4-kb BglII fragment was inserted at the kb +10 EcoRI site of the *H19* locus. Targeting vectors included 7 kb of 5′ homology on a SalI-EcoRI fragment and 3 kb of 3′ homology on an EcoRI-BamHI fragment, a floxed *NeoR* cassette for positive selection, and the *Diphtheria toxin-A* gene for negative selection. After electroporation into embryonic stem cells, G418-resistant clones were screened by Southern blotting with a 1.2-kb BamHI-SalI probe (5′ end) and a 2.1-kb XbaI-BamHI probe (3′ end). If correctly targeted on the 5′ end, H19R cells digested with BamHI enzyme show a 9.0-kb band in addition to the 11.3-kb band indicative of a wild-type chromosome. If correctly targeted on the 3′ end, H19R cells digested with ScaI show a 10.1-kb fragment in addition to the 19-kb fragment indicative of the wild-type chromosome. When digested with ScaI, H19F clones correctly targeted on the 5′ end yield an 11.3-kb band in addition to the 19-kb band indicative of the wild-type chromosome. Clones correctly targeted on the 3′ end show a 7.9-kb ScaI fragment in addition to the 19-kb wild-type ScaI fragment.

To generate *AfpA* and *AfpB*, the *H19DMR* carried on a 2.4-kb BglII fragment was inserted at the kb −0.9 XbaI site of the *Afp* locus. For *AfpD*, the *H19DMR* was carried on a 9-kb BamHI-XbaI fragment. Targeting vectors included 2.5 kb of 5′ homology on a BstEII-XbaI fragment and 3.3 kb of 3′ homology on an XbaI-EcoRI fragment, a floxed *NeoR* cassette for positive selection, and the *Diphtheria toxin-A* gene for negative selection. After electroporation into embryonic stem cells, G418-resistant clones were screened by Southern blotting using the 0.7-kb EcoRI-HindIII probe (5′ end) or the 1.4-kb EcoRI-XbaI probe (3′

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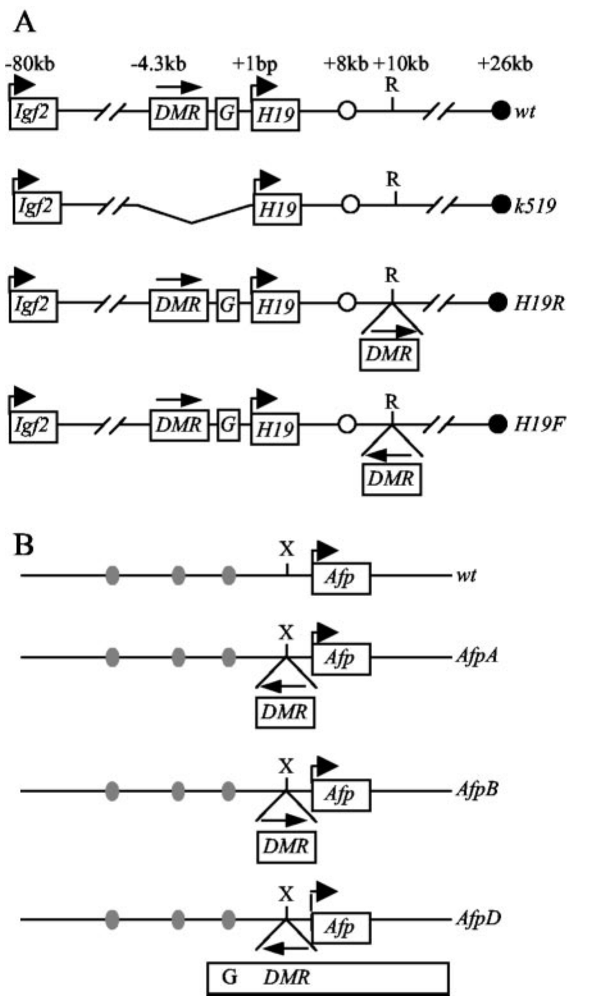

FIG. 1. Schematic diagram of the structures of the chromosomes used in this study. (A) Structures of wild-type (*wt*) and mutant *H19* chromosomes. The *k519* allele carries a deletion of sequences from kb −10 to −0.7 (23). (All numbers are relative to the start site of *H19* transcription.) The *H19R* and *H19F* alleles were generated for this study. These chromosomes each carry a 2.4-kb insertion of the *DMR* on a BglII fragment inserted at the kb +10 EcoRI site (R) and differ only in the orientation of the insert. The endoderm-specific (open circle) and skeletal muscle-specific (filled circle) enhancers are equally functional on both chromosomes (22, 29). (B) Structure of wild-type and mutant *Afp* chromosomes. The *AfpA* and *AfpB* alleles were generated for this study, and each carries a 2.4-kb insertion of the *DMR* on a BglII fragment inserted at the kb −0.9 XbaI site (X). They differ only in orientation of the insert. The *AfpD* allele (generated in this study) has a 9-kb insertion that carries the *DMR* and additional flanking sequences including 461-bp G-rich repeat elements (G). Three enhancers (shaded ovals) and the *Afp* promoter (horizontal arrows) are indicated (38). The 2.4-kb DMR insertion was isolated as a BglII fragment, while the 9-kb insertion was isolated as a BamHI-XbaI fragment.

end). If correctly targeted on the 5′ end, *AfpA* cells digested with EcoRI enzyme yield a 6.3-kb band in addition to the 7.8-kb band indicative of a wild-type chromosome. If correctly targeted on the 3′ end, *AfpA* cells digested with XbaI show a 6.8-kb fragment in addition to the 5-kb fragment indicative of the wild-type chromosome. When digested with EcoRI and with XbaI, *AfpB* clones correctly targeted on the 5′ end yield a 5.8-kb band in addition to the 4.3-kb band indicative of the wild-type chromosome. Clones correctly targeted on the 3′ end show a 7.4-kb XbaI fragment in addition to the 5-kb wild-type fragment. *AfpD* candidates were digested with EcoRI plus XbaI or with XbaI alone to analyze the 5′ and 3′ insertion sites, respectively. Clones correctly targeted at the 5′ end show an 8-kb band in addition to the 5-kb band indicative of the wild-type chromosome, while clones correctly targeted at the 3′ end show 6.8- and 5.0-kb bands indicative of the mutant and wild-type chromosomes, respectively.

Correctly targeted clones were injected into C57/BL6-J blastocysts to generate chimeric founder mice that were mated to *EIIa-cre* transgenic females to generate mice in which the *NeoR* cassette was deleted (28). These mice were identified by PCR amplification across the *NeoR* insertion site. To generate progeny for methylation analysis, mice carrying these mutant chromosomes and a *domesticus* version of the endogenous *Igf2*/*H19* locus were crossed with Dis7CAS mice. Dis7CAS mice are mostly *domesticus* but are homozygous *castaneus* across the *H19*/*Igf2* locus (16). Thus, the Dis7CAS mice provide a wild-type chromosome 7, albeit one that can be distinguished from the wild-type *domesticus* chromosome via multiple DNA polymorphisms. Alternatively, as indicated in the text, mutant chromosomes were introduced into an *H19k519*/*H19k519* background and then backcrossed again to *H19k519*/*H19k519* to generate mice for methylation analysis. The *H19k519* chromosome carries a 9-kb deletion that removes sequences between kb −10 and −0.7, a span that includes the endogenous *DMR* (23).

Bisulfite modification. Genomic DNA was treated with sodium bisulfite according to the manufacturer's recommendation (Intergen). Two micrograms of testes genomic DNA, pooled DNA from 100 blastocysts, or the total DNA of individual embryonic day 7.5 (e7.5) or e8.5 embryos were used in each conversion.

PCR amplification, cloning, sequencing, and restriction analysis of bisulfite-treated DNA. The DNA from approximately 20 blastocysts was used for each PCR. Each subregion was amplified using nested primers essentially as described previously (45). The sequences of the newly designed primers are as follows: subregion 1, BDMRTF5 (5′-TTAGGTATAGTATTTAATGATTTATAAGGG-3′) and BAfpBR3 (5′-AAATACACTATATTTCTAATATAAATTAT-3′), BDMRTF6 (5′ GGGGTGGTATAATATATATTTTTTGGGTAG-3′) and BAfpBR4 (5′-TTGTTTTTATAATCACATCTTTAACATAAC-3′); subregion 2, BDMRTF7 (5′-ATATGGTTTATAAGAGGTTGGAA-3′) and BDMRTR3 (5′-CTACCCAAAAAATATATATTATACCACCCC-3′), BDMRTF8 (5′-TATTTGTGTTTTTGGAGGGGGTT-3′) and BDMRTR4 (5′-CCCTTATAAATCATTAAATACTATACCTAA-3′); subregion 3, BMsp4t1 and BHha4t2, BMsp4t2 and BHha4t3; subregion 4, BHha2t1 and BMsp3t2, BHha2t2 and BMsp3t; subregion 5, BMsp2t1 and BHha1t3, BMsp2t2 and BHha1t4; subregion 6, BDMRTF3 (5′-ATGTAAGTGTGTTTTGTGTAGTAATTGATG-3′) and BMsp1t6, BDMRTF4 (5′-AGATAGTATTGAGTTTGTTTGGAGTTTGAG-3′) and BMsp1t5; subregion 7, BAfpBF1 (5′-TTAAGATGATGATGTTAATAGTAATAAATG-3′) and BDMRTR1 (5′-ACTTTTAACTACATTAAATAAACAATAAAC-3′), BAfpBF2 (5′-GGTATTGATATATTTTTTGATTTTAAGAGTG-3′) and BDMRTR2 (5′-AACTAAACTCCTAATAATTCATTTACATTT-3′). The PCR products were cloned using a TA cloning kit (Invitrogen), and the clones were sequenced on both strands. Alternatively, the PCR product was purified and restricted with AciI enzyme.

RESULTS

To test the ability of the *DMR* to mark the chromosome differentially in a parent-of-origin-dependent manner, we inserted it at two locations in the genome: at the kb +10 position of the *H19* gene and at kb −0.9 of the *alpha-fetoprotein* gene (*Afp*) (Fig. 1). We believe that the kb +10 position is past the 3′ boundary of the imprinted cluster on chromosome 7 because no molecular marks distinguishing maternal and paternal chromosomes have been documented in the region. Moreover, the enhancer elements centered at kb +8 and at kb +24 are each fully functional on both maternal and paternal chromosomes (22, 29). Nonetheless, the kb +10 position is clearly proximal to other sequences that may normally contribute to marking the parental origin of the *Igf2*/*H19* alleles. Insertion at the *Afp* locus on chromosome 5 is therefore a more stringent test of the sufficiency of the *DMR* to act as an *ICE*. The *Afp* gene is expressed biallelically (data not shown), and there are no known imprinted genes on mouse chromosome 5 (www.mgu.har.mrc.ac.uk/research/imprinted/imprin.html).

The *Afp* locus has several advantages for our study. First, mice heterozygous for loss-of-function mutations show no discernible phenotype (15). Second, the regulated expression of mouse *Afp* has been extensively studied in vitro and in vivo using transgenic animals (38). These studies have identified three upstream enhancers as well as promoter elements that can account for activation of the *Afp* gene (Fig. 1B). Insertion of the *DMR* at the kb −0.9 position at *Afp* puts the *DMR* just upstream of the promoter, thus mimicking its location at the *H19* locus. However, this same positioning also mimics the organization of the *Igf2* gene in that the *DMR*, with its insulator activity, now separates the *Afp* promoter and enhancer elements.

The *Afp* locus is not a CpG-rich region. Specifically, there are no CpG islands in the enhancer or promoter regions or in any sequences within 50 kb upstream and 14 kb downstream of the *DMR* insertion site (http:ccnt.hsc.usc.edu/cpgislands). (The region 14 kb downstream of the insertion site is defined as a CpG island only when using the least stringent criteria.) At its endogenous location, the *DMR* is proximal to a CpG island that includes the *H19* promoter and extends into the *H19* RNA coding sequences. There is no equivalent sequence motif at the *Afp* locus. For example, the 0.9-kb sequences downstream of the insertion site on chromosome 5 (which include the *Afp* promoter) contain 10 scattered CpGs.

We isolated the *DMR* as a 2.4-kb BglII fragment. This fragment carries the 65 CpG base pairs that are methylated in sperm and escape demethylation during early embryogenesis. These sequences include all four binding sites for CTCF, a protein that is crucial for normal transcriptional regulation of the *Igf2*/*H19* gene cluster, but the BglII fragment does not include the promoter-proximal G-rich repeats. At each chromosomal locus, the *DMR* was inserted in both orientations to generate mutant chromosomes *H19R*, *H19F*, *AfpA*, and *AfpB* (Fig. 1).

We generated founder lines by injecting mutated embryonic stem cells into wild-type blastocysts. The *NeoR* cassettes used for positive selection in vitro were removed by crossing these founders to females carrying a *Cre* recombinase gene under the control of the *EIIa* promoter (28). Male and female progeny of these crosses were then mated with wild-type tester mice to generate pups for analysis. Our crosses were set up so that pups inherited the *DMR* insertion and also a *domesticus* wild-type copy of the endogenous *DMR* from one parent while inheriting a *castaneus* wild-type version of the endogenous *DMR* from the other parent. Given the polymorphisms that distinguish *castaneus* and *domesticus DMR* alleles and the polymorphisms generated by the different sequences flanking the *DMR* in its normal and heterologous positions, we could distinguish all three *DMRs* in each pup: endogenous paternal *DMR*, endogenous maternal *DMR*, and heterologous *DMR*.

We tested for cytosine methylation of AciI sites in DNAs isolated from somatic tissues of postnatal animals and found that the inserted *DMRs*, just like the endogenous copy, are methylated when paternally inherited but not when maternally inherited (Fig. 2A, top panel). This property is orientation independent for both insertion locations. These results demonstrate that the *DMR* contains sufficient information to mark its own parental origin, even on a heterologous chromosome in a nonimprinted genomic context.

At its normal position, the *DMR*, whether paternal or maternal, is methylated in mature sperm. In fact, demonstration of the acquisition of methylation on both chromosomes during spermatogenesis is vital empirical support for the notion that parent-of-origin-specific methylation is the primary (or gametic) imprint of the *H19*/*Igf2* locus (46). We wished to determine whether this mechanism would apply to the mutant chromosomes and therefore isolated DNAs from the testes of sexually mature males and assayed for methylation at AciI sites in the *DMR* inserts (Fig. 2A, bottom panel). At the *H19* locus, the *DMR* insert is always completely methylated and thus behaves identically to the endogenous maternal and paternal *DMR* alleles. In contrast, the *DMR* insert at the *Afp* locus is not methylated. We confirmed and extended these results by digesting with other methylation-sensitive enzymes, including HhaI, ClaI, BspEI, and HpaII (data not shown but see Fig. 5 for maps), which together assayed methylation at 24 CpG sites within the *DMR*. To examine all 65 CpGs, we examined the methylation status of several testis samples by direct sequencing (Fig. 3A). To assay the entire insert region, we required seven nested-PCR amplifications. Each amplification was done on two independently prepared DNA samples, and multiple clones were analyzed for each reaction. The results confirmed that there is no consistent cytosine methylation of the *DMR* insert in adult testis.

In sum, the paternal specific cytosine methylation found on *Afp*::*DMR* inserts in differentiated tissues cannot be explained by maintenance of a difference inherited through the germ cells. Rather, our results imply that when inserted at the *Afp* locus, the *DMR* is marked differentially in the two gametes by a mechanism other than its own DNA methylation and that the differential DNA methylation across the *DMR* is acquired as a secondary imprint later in the development.

To determine when the methylation at the mutant *Afp* loci is acquired, we examined various developmental stages for parent-of-origin-specific methylation of the *DMR*. First, we assayed methylation at the blastocyst stage. We converted DNA isolated from pools of approximately 100 blastocysts and then used one-fifth of this DNA for each PCR. We independently amplified DNA two to four times for each PCR primer set and then analyzed multiple clones for each reaction. Our results showed that paternally inherited *DMRs* are largely unmethylated at this stage of development (Fig. 3B) even though control sequences (the maternal *Snrpn* locus and the paternal endogenous *H19DMR*) from the same DNA samples were methylated as expected (data not shown). The lack of the DNA methylation in the blastocyst confirms that the methylation of the *DMR* is not the primary imprint.

We next tested restriction enzyme sensitivity of PCR-amplified samples to examine methylation of DNAs isolated from pooled blastocysts and also from individual e7.5 and e8.5 embryos. The sequences we examined included two of the four CTCF binding sites. Paternal *DMRs* from gastrulated embryos but not from blastocysts were always methylated at least partially (Fig. 4). Finally, we examined DNAs isolated from e11.5 and e12.5 embryos by using Southern analyses and determined that methylation of the *DMR* was complete and not distinguishable from that seen in adult tissue samples (data not

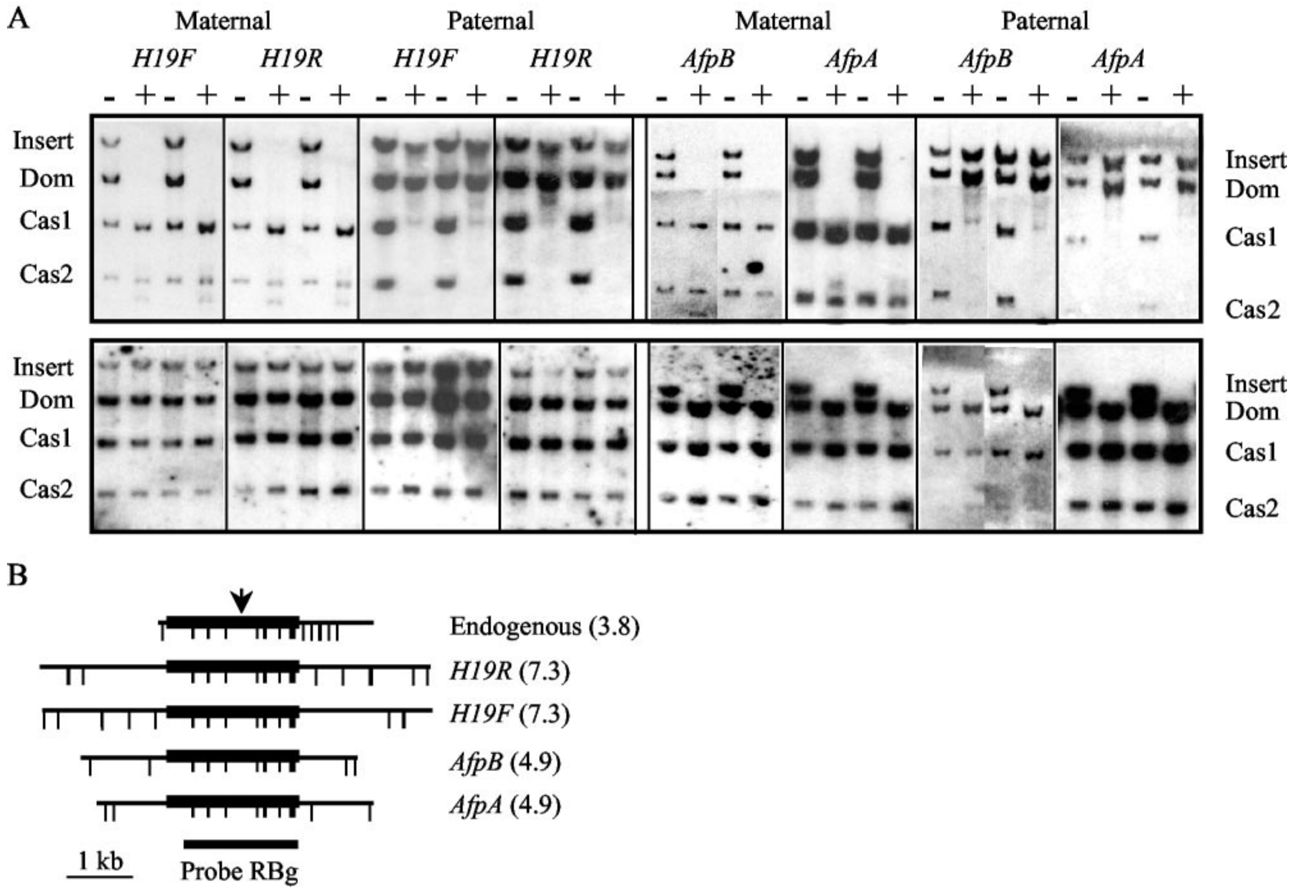

FIG. 2. DNA methylation of the *H19DMR*. (A) DNAs isolated from kidney (top panel) or testis (bottom panel) were digested with SacI (−) or with SacI plus AciI (+) and analyzed by Southern blotting. The identity of the *DMR* insertion and its parental origin are indicated above the lanes. At the endogenous *H19* locus, all mice carry one wild-type *domesticus* allele and one wild-type *castaneus* allele of the *DMR*. Mice were generated such that the *domesticus* allele is always inherited from the same parent as the *DMR* insert. Insert, the SacI fragments associated with the *DMR* insertions at *H19* and *Afp* are 7.3 and 4.9 kb, respectively; Dom, 3.8-kb SacI fragment associated with the endogenous *domesticus DMR*; Cas1 and Cas2, 2.3- and 1.5-kb SacI fragments, respectively, associated with the endogenous *castaneus DMR*. The *castaneus* allele yields two bands upon SacI digestion because of the polymorphic SacI. (B) AciI restriction maps of the SacI fragments carrying the endogenous *DMR* and the *DMR* inserts downstream of the *H19* gene and at the *Afp* locus. The arrow above the top line indicates the polymorphic SacI site unique to the wild-type *castaneus DMR*. The 1.8-kb EcoRI-BglII probe used to identify the *DMR* is indicated.

shown). Thus, the acquisition of parent-of-origin-specific methylation differences occurs after implantation and around the time of gastrulation. This is the time period when large parts of the genome, including most CpG islands, are undergoing methylation (36). Our results are consistent with two possibilities: (i) the maternally inherited *DMR* is refractory to de novo methylation or (ii) the paternally inherited *DMR* attracts such methylation.

DMR insertions at the *H19* locus (endogenous and kb +10) are methylated in sperm while those at the *Afp* locus are not. We wished to see whether we could isolate DNA sequences responsible for this difference and therefore generated a larger insertion at the *Afp* locus (Fig. 1). Specifically, this new insert, *AfpD*, contains sequences from kb −10 to −0.7 upstream of the *H19* gene and thus carries, in addition to the *DMR*, the G-rich repeat elements that are common to many imprinted genes. However, this larger insert behaves similarly to the 2.4-kb *DMR* element. That is, the paternally inherited insert is hypermethylated in differentiated tissues (data not shown) but not methylated in sperm (Fig. 5).

DISCUSSION

Loss-of-function mutations in the mouse suggest that the sequences encompassing the *H19DMR* are necessary for at least three genetic functions that are each crucial for maintaining the normal monoallelic expression patterns characteristic of this locus. First, these sequences carry a methylation-sensitive transcriptional insulator whose normal function prevents activation of the *Igf2* gene by the downstream enhancers it shares with the *H19* gene (2, 19, 23–25, 41). Gain-of-function analyses have confirmed that the *DMR* sequences are also sufficient for this activity, at least in vitro (2, 19, 20, 23–25). Second, a paternally inherited and marked *DMR* acts as a developmentally regulated silencer that induces in *cis* stable changes at the *H19* locus that maintain silence of the paternal

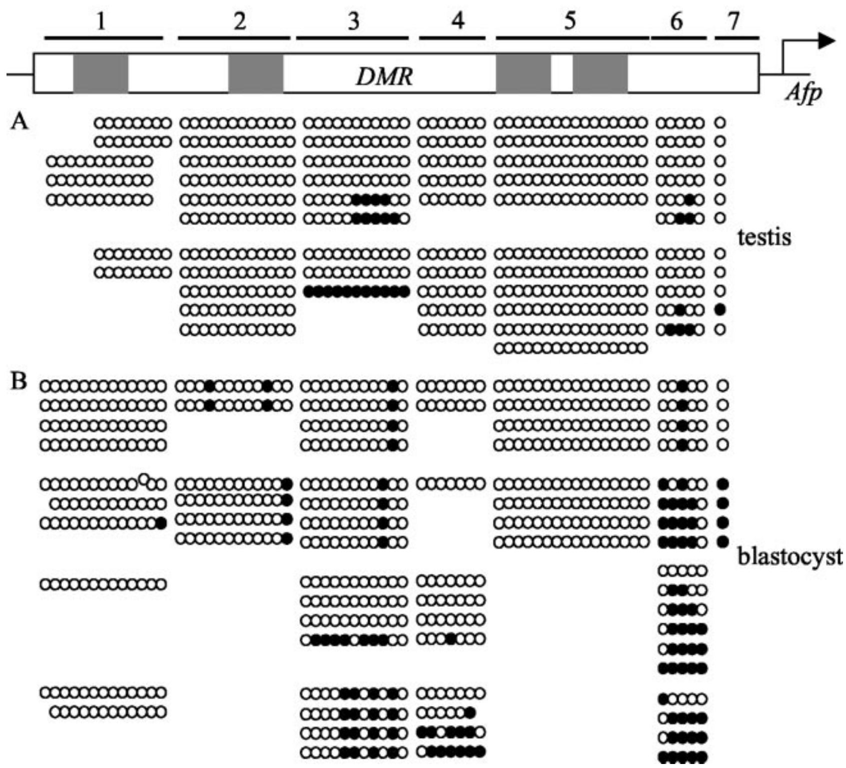

FIG. 3. Cytosine methylation of the *H19DMR* when inserted at the *Afp* locus as measured by direct sequencing of bisulfite-treated DNA. Methylated (filled circles) and unmethylated (open circles) CpG dyads are displayed. Bisulfite-treated DNAs were amplified in seven distinct PCRs (lines at top), and the PCR products were cloned and sequenced. The locations of the CTCF binding sites are indicated by the shaded boxes. (A) Methylation of paternally inherited *AfpA DMRs* in adult testes. DNAs were extracted from testes of two *H19k519*/*H19k519 Afp*$^{+}$/*AfpA* mice and analyzed for CpG methylation. Two to six clones were sequenced for each testis sample. (B) Methylation of paternally inherited *AfpA DMRs* in blastocysts. DNA was extracted from pools of 100 *H19k519*/*H19k519 Afp*$^{+}$/*AfpA* blastocysts and treated with bisulfite. About one-fifth of this DNA (20 blastocysts) was used in each PCR. Multiple reactions were performed for each subregion as indicated by the spaces between clusters. For example, PCR 1 was performed four times on unique pools of converted DNA and then four, three, one, and two clones were obtained from these reactions and individually sequenced. Because of the limiting starting materials and the destruction of the DNA that is inherent in the bisulfite treatment, only clones from separate PCRs are certain to represent distinct chromosomes.

allele (39, 40). Third, the results of the genetic studies were consistent with the notion that the *DMR* acts as an *ICE*. In this context, we define the *ICE* narrowly and mean only the *cis* sequences whose gametic epigenetic marking distinguishes the maternal and paternal chromosomes.

Our results demonstrate first that the 2.4-kb *DMR* is in fact an *ICE*. The *DMR* sequences maintain the ability to keep track of their parental origin even on a heterologous chromosome. Although the *H19* and *Igf2* genes are part of a very large (>1 Mb) imprinted domain, the functional differences noted between the paternal and maternal alleles of these two genes can be ascribed to a highly localized signal contained on a mere 2.4-kb sequence. The *DMR* sequence carries information that results in its being methylated only when paternally inherited. Because sequences within the *DMR* act as a methylation-sensitive insulator and silencer, this methylation induces transcriptional differences in paternal and maternal chromosomes. At its endogenous location on chromosome 7, the methylated *DMR* allows expression of paternal *Igf2* by inactivating the transcriptional insulator that comaps with the *ICE*. The methylated *DMR* conversely blocks expression of paternal *H19* by acting as a developmentally regulated silencer (37, 43). In fact, we, of course, analyzed transcription of *Afp* in our mutant mice and noted a fivefold parent-of-origin effect on transcription (Sangkyun Jeong and K.P., unpublished observations).

However, interpretation of these transcription results is not straightforward. Given the topology of the *Afp* locus, we introduced the *DMR* into a position where it potentially operates as an insulator on the unmethylated maternal chromosome but as a silencer on the methylated paternal chromosome (Fig. 1). Such a dual effect of the insertion on *Afp* transcription is consistent with the results we actually obtained: maternal inheritance lowering expression about fivefold and paternal inheritance lowering expression about 25-fold compared with wild-type levels. However, a real understanding of the transcriptional effects of the insertions will require the analysis of several additional control chromosomes that we are presently generating. These new mutations will allow us to distinguish silencing from insulation and also to quantitate any effects on promoter activity that are due to shifting the distance between the *Afp* promoter and its upstream enhancers.

Localization of the parent-of-origin identification entirely to the *DMR* clarifies the complex and sometimes perplexing analysis of *H19* transgenes generated by pronuclear injection. The earliest transgenic studies demonstrated a critical importance for the *DMR* but also showed that other sequences such as the

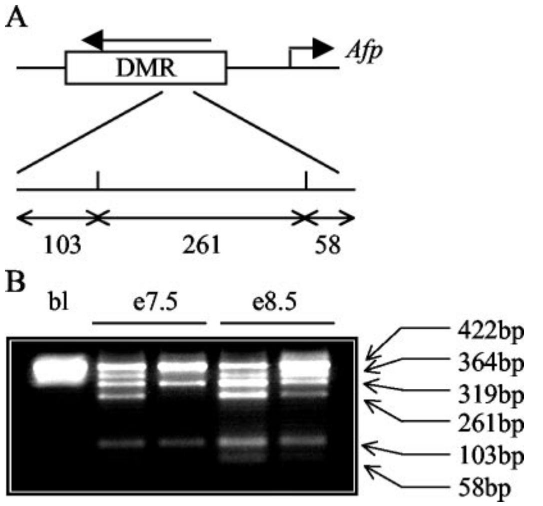

FIG. 4. Developmental changes in the DNA methylation of the *AfpA DMR* insert. (A) Summary of the assay. The digestion of two AciI sites (vertical bars) within the PCR product of subregion 5 of DMR depends on the methylation status of the genomic DNA. If the CpGs in the AciI sites are methylated in the genomic DNA, they remain as CpG (unconverted) after the treatment with sodium bisulfite; hence, the PCR product can be digested with AciI. If those CpGs are unmethylated in the genomic DNA, they will be converted to TpG by treatment with sodium bisulfite and the PCR product will be insensitive to AciI. These AciI sites each represent a CTCF binding site. (B) Results of AciI digestion. DNAs isolated from pooled blastocysts (bl) or from individual e7.5 and e8.5 embryos were converted, amplified, digested with AciI enzyme, and analyzed by gel electrophoresis. Embryos were Afp^{+}/*AfpA H19k519*/*H19k519*; thus, no endogenous copies of the DMR were present.

enhancer elements were essential (11, 35). These same studies also indicated that copy number was critical. More recent investigations using 5′ sequences that include the entire *DMR* actually showed that even single-copy *H19* transgenes can be imprinted (5, 23). It seems plausible that the apparent reliance on *H19* sequences outside the *DMR*, like the apparent reliance on multiple transgene insertions, was noted only because the so-called *DMR* was in fact a shortened (i.e., mutated) version.

The second major finding in this report is that the epigenetic imprinting marking of the *DMR* and cytosine methylation of the *DMR* are separable. In other words, at least with the *DMR* insert at the *Afp* locus, the primary imprint does not appear to be its DNA methylation. Rather, differential methylation is established after implantation by a mechanism that is not yet understood but presumably as a result of an interpretation of the true primary mark. Our results do not imply that DNA methylation does not play a critical role in parent-of-origin-specific expression or in imprinting even at the *Afp*::*DMR* locus but suggest that methylation of the *DMR* itself is not the obligatory gametic mark.

We examined *Afp* sequences for CpG-rich regions that might play a surrogate imprinting role on our chimeric chromosomes. As described in Results, we did not note any nearby CpG islands. The 0.9 kb between the DMR insertion and the *Afp* transcriptional start site contains 10 CpGs, including four dyads whose methylation status could be evaluated by restriction digestion and Southern blotting. Our initial analysis did not reveal any consistent methylation patterns to distinguish maternal from paternal chromosomes or wild-type from insertion chromosomes (Sangkyun Jeong and K.P., unpublished observations).

A key question that our present study cannot directly address is whether the same primary mark that ultimately establishes parent of origin at the *Afp*::*DMR* locus also applies to the endogenous *DMR*. In other words, is the difference between the functions of the *DMR* at its normal position and at the *Afp* locus only the timing of when the primary mark is converted to differential methylation? Alternatively, does the insertion of the *DMR* at the *Afp* locus create a completely novel mechanism for genomic imprinting?

Our results recall those of El-Maarri et al. (10), who examined methylation patterns for the *SNRPN* locus in human oocytes. Although they started with very limiting material and were not able to identify the *ICE* as a sufficient element for the imprinting, these investigators did not detect maternal specific methylation and thus suggested that heritable alterations other than DNA methylation might mark maternal and/or paternal alleles.

However, our results do not fit well with those of another important 2001 study (Howell et al. [21]), which examined the role of the oocyte-specific isoform of the *DNA* methyltransferase gene 1 (*Dnmt1o*). *Dnmt1o* is a maternal effect gene. Females homozygous for a *Dnmt1o* deletion are fine, but fetuses from such mothers do not survive and show loss of imprinting. Specifically, for example, *H19* expression becomes biallelic and one half of all paternal chromosomes show complete loss of methylation while the other half show the normal methylated pattern. (Likewise, *Snrpn* becomes biallelic, with half of the maternal chromosomes aberrantly showing a complete loss of methylation.) Given the protein expression and localization patterns, Howell et al. explained these results by postulating that the Dnmt1o isoform is required specifically at the eight-cell morula stage to maintain methylation during cell division. This interpretation implies that the paternal marking of the endogenous *H19DMR* (and *Snrpn*) is dependent on DNA methylation even before implantation.

The nature of the primary mark on the *DMR* insert at *Afp* is presently unknown. Besides DNA methylation, additional differences in the chromatin structure of *H19DMR* on each chro-

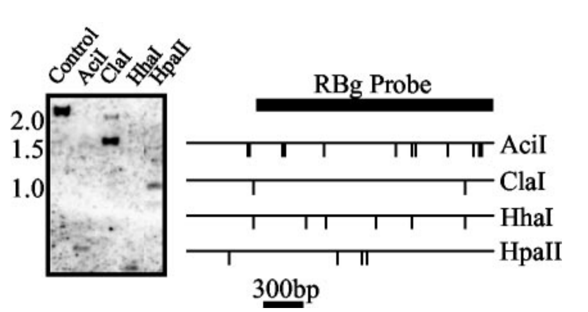

FIG. 5. Methylation of the *AfpD DMR* insert. DNAs prepared from adult testes were digested with BglII alone (Control) or with BglII plus AciI, BglII plus ClaI, BglII plus HhaI, or BglII plus HpaII and analyzed by Southern blotting. Size markers (in kilobases) are indicated at far left. Restriction maps of the 2.4-kb BglII fragment for each of the digests are displayed to the right along with the 1.8-kb EcoRI-BglII probe. The mice for this experiment were *H19k519*/*H19K519*, so both copies of the endogenous *DMR* were deleted.

mosome have been previously reported (1, 14, 18, 26, 27, 42). However, these parent-of-origin-specific differences in nuclease sensitivity and in histone codes were only characterized in tissues which also showed differential methylation, thus making it impossible to distinguish the cause-and-effect relationships of these potential marks. Davis et al. (6) examined the acquisition of DNA methylation at the endogenous *H19DMR* during spermatogenesis. They noted that the distinctive methylation of the *H19DMR* was acquired in a two-step process. First, all methylation was removed from the paternal chromosome, and then both maternal and paternal *DMRs* were remethylated. Their experiments demonstrated that, even without cytosine methylation, the maternal and paternal chromosomes were functionally nonequivalent because the paternal chromosome was remethylated earlier than the maternal. However, these experiments could not clarify whether the difference implied the existence of a primary imprint other than DNA methylation or it implied only that secondary chromatin changes caused by differential DNA methylation can remain for a while even after the erasure of that methylation. Further characterization of the epigenetic modification by using the system reported here will clarify the role of nonmethylation epigenetic marks and help illuminate the general mechanisms by which the genome is imprinted.

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